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Award Number: W81XWH-05-1-0063

TITLE: The Role of hCDC4 as a Tumor Suppressor Gene in Genomic Instability Underlying Prostate Cancer

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REPORT DATE: November 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-11-2005	Annual Summary	1 Nov 2004 – 31 Oct 2005

4. TITLE AND SUBTITLE
The Role of hCDC4 as a Tumor Suppressor Gene in Genomic Instability Underlying Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0063

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Sidney Kimmel Cancer Center
San Diego, CA 92121-1131

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This study investigates the role of a newly identified gene called *hCDC4* in prostate cancer. The *hCDC4* gene encodes a protein that functions in a cellular process called proteolysis, or protein degradation. hCdc4 degrades a protein called cyclin E1, which is a central component of the cell division machinery. Cyclin E1 is involved in initiating DNA replication in cells. However, in many types of human tumors cyclin E1 protein levels are aberrant and this phenotype has been shown *in vitro* and *in vivo* to be oncogenic. Very little is known regarding cyclin E/hCdc4 in prostate tumors.

We are exploring whether *hCDC4* functions as a tumor suppressor gene in prostate cancer. We have completed a genetic screen of prostate tumors and found an *hCDC4* gene mutation. We have shown that this mutant hCdc4 cannot bind cyclin e1 substrate in vivo. We are currently determining whether *hCDC4* functions as a haplo-insufficient tumor suppressor through LOH and expression analysis.

15. SUBJECT TERMS
hCDC4, tumor, prostate

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	7	

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Introduction

This study investigates the role of a newly identified gene called *hCDC4* in prostate cancer. The *hCDC4* gene encodes a protein recently identified as an F-box protein that functions in a cellular process called proteolysis, or protein degradation. hCdc4 degrades a protein called cyclin E1 (Strohmaier, H. *et al*. 2001; Moberg, K.H. *et al*. 2001; Koepp, D.M. *et al*.2001). Cyclin E1 is involved in initiating DNA replication in mammalian cells. Cyclin E1 abnormalities have been reported in many types of human tumors (Sandhu, C. and Slingerland 2000). Evidence implicating a role for deregulated cyclin E1 associated kinase activity in prostate tumorigenesis is suggested through studies of the cyclin E1/Cdk2 inhibitor p27. In prostate tumors, p27 protein levels are low or absent and this phenotype is associated with poor patient prognosis (Macri, E. and Loda, M. 1998). In this proposal we explore whether *hCDC4* functions as a tumor suppressor gene in prostate cancer through its role in cyclin E1 proteolysis.

Body

Task 1- Determine the role of hCDC4 as a tumor suppressor in prostate cancer (Months 1 -20)

a. ***Identify and isolate fresh-frozen and/or archival prostate tumor specimens from the tissue bank at The Sidney Kimmel Cancer Center (Months 1-2).***

We have obtained 40 prostate tumor specimens from the SKCC Tumor Bank. Four sections of 10 μm thickness were obtained for each fresh-frozen tumor specimen.

b. ***Isolate DNA, RNA and protein from fresh frozen prostate tumor specimens (Months 2-3)***

Two 10 μm sections of each tumor specimen were used for DNA isolations using the QiaAmp DNA Isolation Kit (Qiagen). Approximate total yield of DNA for each sample was 20 μg. Each DNA sample was diluted to yield a 20 μg/ml stock solution.

Two additional tumor specimens were lysed in mammalian RIPA lysis buffer and cellular proteins extracted. A total yield of approximately 100 μg protein was obtained/sample.

c. ***Microdissect matching normal DNA tissue from paraffin-embedded archival tissue specimens (Months 2-3)***

Normal tissue for each tumor specimen was marked by microscopic examination and microdissected under a light microscope (mag x40). DNA was isolated by standard proteinase K digestion. DNA will be used as control in loss of heterozygosity (LOH) determinations.

d. ***Screen prostate tumors for hCDC4 gene mutations by SSCP (Months 3-6)***

We have screened 40 prostate tumor specimens for *hCDC4* gene mutations by SSCP. Eighteen different PCR reactions were used to cover the 13 different exons of the *hCDC4* gene. An aberrant SSCP banding pattern was detected for a single prostate tumor specimen corresponding to the α-exon of *hCDC4* (Fig. 1).

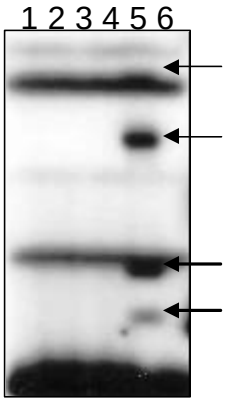


Figure 1. ***hCDC4 gene mutation in a prostate tumor.*** **SSCP analysis of the a-exon of *hCDC4* demonstrated an aberrant banding pattern for tumor in lane 5 (arrows). DNA sequencing revealed the mutated allele contains a three base pair insertion (CCG) introducing an in-frame proline residue in the hCdc4 protein (see below).**

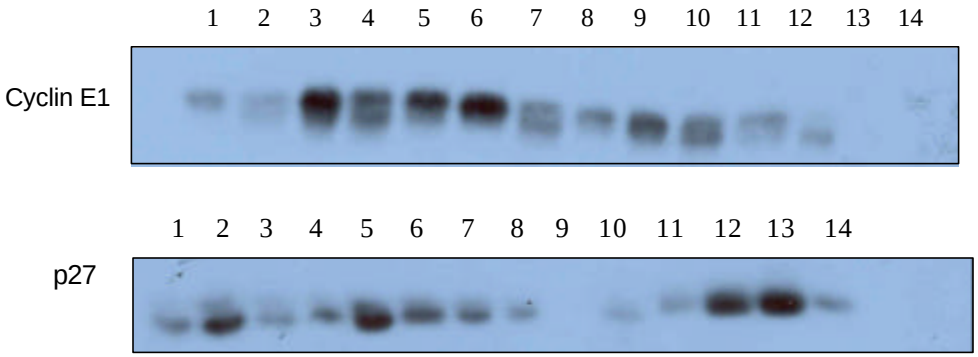
e. ***Sequence hCDC4 gene mutations (Months 5-6)***

We cloned the *hCDC4* alpha-exon for the prostate tumor containing an aberrant SSCP banding pattern in pCRII-TOPO (Invitrogen). DNA sequencing revealed a three base pair insertion in the gene. This sequence is predicted to introduce an in-frame proline residue in the hCdc4 protein.

f. ***Western blot analysis of cyclin E and hCdc4 protein in prostate tumor specimens (Months 8-10)***

We have isolated protein from 40 fresh frozen prostate tumor specimens. Approximate yield of protein for each sample was 100 µg. We have performed Western blot analysis of all 40 prostate tumor specimens suing antibodies specific for cyclin E1 and p27 (Fig. 2). As expected, we found that numerous prostate tumors contained an elevated level of cyclin E1 protein. Additionally, numerous tumors contained a low or absent level of p27.

Figure 2. Western blot analysis of cyclin E1 and p27 in prostate tumors specimens obtained from the tissue bank at The Sidney Kimmel Cancer Center.



We have not been able to analyze hCdc4 protein due to the lack of an anti-hCdc4 antibody that gives a sufficiently low background by Western blot analysis. These results have necessitated our undertaking

of LOH and Real-time PCR analysis to substitute for hCdc4 western blot analysis in hCdc4 expression determinations.

g. *Immunohistochemical staining of archival prostate tumor specimens for cyclins E, A and B1 (Months 9-12)*

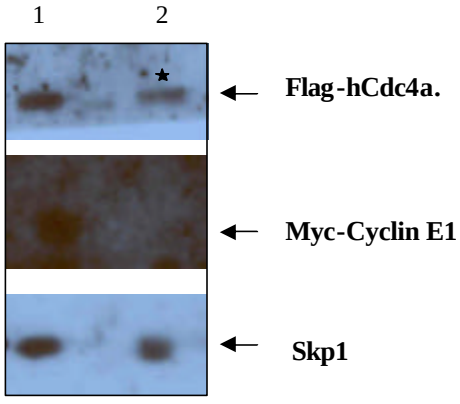
We have immuno-histochemically analyzed prostate tumor specimens containing an *hCDC4* mutation or wild-type alleles. Archival paraffin-embedded specimens were analyzed for cyclin E1 and cyclin A expression. Slides were analyzed microscopically for the percentage of positive staining nuclei. Determinations for cyclin E1 were found to be difficult to interpret due to high background. We are currently exploring alternative fixation and detection methods in order to limit background staining.

h. *Functional analysis of mutant hCDC4 alleles (Months 9-12).*

We cloned the mutant *hCDC4* alpha-exon for the prostate tumor containing an aberrant SSCP banding pattern in pCRII-TOPO (Invitrogen). DNA sequencing revealed a three base pair insertion in the gene. This sequence is predicted to introduce an in-frame proline residue in the hCdc4 protein.

To test whether this mutation causes an alteration in hCdc4 function, we tested its ability to complex with cyclin E1 *in vivo*. We constructed mammalian expression vectors that express wild-type or mutant hCdc4 tagged with the Flag epitope. We co-transfected human embryonic kidney 293T cells with vectors that express wild-type or mutant hCdc4, together with myc-tagged cyclin E1. To determine whether the mutant hCdc4 can complex with the SCF core components, we also co-transfected cells with a Skp1 expression vector. Protein complexes were isolated using anti-Flag agarose and proteins separated by SDS-PAGE. As shown in Fig. 3, the wild-type hCdc4 was shown to bind cyclin e1 but the mutant hCdc4 could not. These results show that the *hCDC4* mutation found in the prostate tumor destroys the substrate recognition function of hCdc4.

Figure 3. Mutant hCdc4 protein cannot bind cyclin E1 substrate in vivo. 293T cells were co-transfected with expression plasmids for Skp1, myc-cyclin E1, together with wild-type hCdc4 (lane 1) or mutant hCdc4 (lane 2). Extracts were then immunoprecipitated using anti-Flag agarose.



Key Research Accomplishments

1. We have detected the first *hCDC4* gene mutation in a prostate cancer.
2. We have analyzed cyclin E1 and p27 in prostate tumor specimens.
3. We have found that a mutant hCdc4 protein found in prostate tumors cannot complex with cyclin E1 substrate in vivo.

Reportable Outcomes

The data obtained upon completion of this project will undoubtedly necessitate the publication of a manuscript. The results of this study have prompted us to apply for a DOD Prostate Award to fund a continuation of this research. This proposal will analyze *hCDC4* defects in more detail and determine the role of hCdc4 in genetic instability and androgen-independent proliferation in prostate tumorigenesis.

Conclusions

We have discovered that the *hCDC4* gene functions as a tumor suppressor in prostate cancer. *hCDC4* inactivation/cyclin E1 deregulation may be a major cause of genetic instability and androgen-independent proliferation of prostate tumor cells.

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Appendices

N/A